

Paralytic Peptide for Use in Neuromuscular Therapy

Cross Reference to Related Application:

This application claims priority from United States application no. 60/427,682, filed November 18, 2002, which is incorporated by reference herein in its entirety.

5 Field of the Invention

The invention relates to a paralytic peptide for neuromuscular therapy and other uses requiring disruption of neuromuscular mechanisms.

Background of the Invention

10 Shrews are a very ancient group of primitive mammals that resemble most closely the proto-mammals. They are not closely related to rodents which evolved from different groups of mammals. According to Dufton (1992), the known venomous species of shrew are: the northern short-tailed shrew (*Blarina brevicauda*), the Haitian solenodon (*Solenodon paradoxus*), the European water shrew (*Neomys fodiens*) and the Mediterranean shrew (*Neomys anomalus*). Another venomous shrew is the southern
15 short-tailed shrew (*Blarina carolinensis*). It has also been suggested that the Cuban solenodon (*Apotogale cubanus*) and the American shrew (*Sorex cinereus*) could be venomous. The northern short-tailed shrew (*Blarina brevicauda*) and its closely related species use a paralytic venom in its saliva to paralyze insects, other invertebrates (worms, annelids etc.), nesting birds and small mammals which it then stores, alive in its den, for
20 future feeding (Martin 1981; George et al. 1986; Dufton 1992).

The shrew venom literature generally consists of seven articles from the 40s and 50s and one MA thesis in 1966 [Christenbury 1966]. These are summarized in a review [Dufton 1992]. Using a crude ammonium sulfate precipitate of shrew saliva glands, Ellis and Krayner (1955) concluded the active agent was probably a protein and, because of its
25 inability to dialyze, a larger protein. A major contribution of the Ellis & Krayner work was to show activity in cats, dogs, mice, rats, guinea pigs and rabbits. Christenbury

[1966] showed Ellis & Krayner's preparation stopped oxygen consumption by mouse kidney and liver slices. Japanese patent application (JP 10-236963; 1998) appears to disclose an alcoholic extract of saliva glands from two shrew species (*Sorex unguiculatus* & *Sorex shinto saevus*) as a calcium channel blocker and its use as a hypotensive. The
5 purity is low – the extract includes any compounds that would dissolve in 70% ethanol. There is no information about the responsible active molecule/s in the unknown mixture of compounds.

Summary of the Invention

The paralytic compound of shrew saliva remained unidentified until now. The inventors
10 have isolated and purified a paralytic compound having the sequence shown in Figure 1A (SEQ ID NO:1) or Figure 1B (SEQ ID NO:2). The inventors further show that, while a high molecular weight fraction is paralytic, the active molecule is not a large protein but, unexpectedly, a small peptide bound in a large complex of many proteins (**Fig. 3, Lane 1**). The invention relates to a low molecular weight peptide (or optionally a suite of
15 related peptides), preferably, isolated and purified from the submaxillary saliva glands of shrews of a species such as *Blarina* as a paralytic agent. All or part of the peptide or its parent pro-peptide may also be produced by recombinant DNA methods or *in vitro* or *in vivo* peptide synthesis. This novel paralytic agent is useful as a neuromuscular blocker.

As mentioned above, the active ingredient is a small peptide isolated in an unusual and
20 unexpected combination within a large protein complex. Known mammalian saliva peptides (e.g. vasoactive intestinal polypeptide & glucagon-like peptide1 [Pohl & Wank 1998]) would not be contaminants as they are discarded with inactive, low molecular weight molecules during the purification protocols. The preparation of the invention is of great purity and can be extracted from an unexpected sub-cellular source.

25 The present inventors have isolated and purified novel proteins from the submaxillary saliva glands of shrews. In accordance with one embodiment of the invention, there is provided an isolated and purified shrew saliva peptide. In a specific embodiment, the isolated and purified shrew saliva peptide has the amino acid sequence shown in **Figure**

1A or 1B. The invention includes methods of isolating a paralytic compound from venomous shrew saliva gland or shrew saliva, comprising providing the gland or saliva, isolating the paralytic compound from the gland or saliva and optionally purifying the compound.

5 The present invention also provides a pharmaceutical composition or a cosmetic composition that includes the isolated and purified shrew saliva peptide, and the use of the peptide as a pharmaceutical substance, neuromuscular blocker or an analgesic. The invention is yet further directed to the use of the isolated and purified shrew saliva peptide for treatment of migraine, myofacial and other types of pain, muscle tremors,
10 neuromuscular diseases, excessive sweating and wrinkles.

In particular, the invention is directed to a method of preventing or treating migraines, myofacial and other types of pain, muscle tremors, neuromuscular diseases, and excessive sweating in a mammal comprising administering to the mammal an isolated and purified shrew saliva peptide, for example in a pharmaceutical composition. The
15 mammal is preferably a human. The invention is also directed to a method of providing analgesia or neuromuscular blocking in a mammal comprising administering to a mammal a pharmaceutical composition including the isolated and purified shrew saliva peptide. The invention is further directed to a method of preventing or reducing wrinkles in a mammal comprising administering to the mammal the isolated and purified shrew
20 saliva peptide, for example in a cosmetic composition.

The invention is also directed to the use of the isolated and purified shrew saliva peptide for the preparation of antibodies, including polyclonal antibodies, monoclonal antibodies or functional fragments thereof. This invention also relates to the antibodies so produced.

The invention is yet further directed to a method of determining the potency of a paralytic agent by administering the paralytic agent to a mealworm or other insect; determining the
25 time until onset of paralysis and/or the duration of paralysis; and wherein the time for onset of paralysis is inversely proportional to the strength of the paralytic agent and the duration of paralysis is proportional to the strength of the paralytic agent.

Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications
5 within the spirit and scope of the invention will become apparent to those skilled in the art from the detailed description.

Brief Description of the Drawings

These and other features of the invention will become more apparent from the following
10 description in which reference is made to the appended drawings wherein:

Fig. 1. Amino acid sequences of isolated and purified shrew saliva protein: A. (SEQ ID NO:1); B. (SEQ ID NO:2)

Fig. 2. Size exclusion chromatography of shrew submaxillary gland extract with bioactive fractions indicated by cross-hatching.

15 Fig 3. SDS-PAGE analysis of shrew submaxillary gland extract. The small active component exists as part of a very high molecular weight complex.

Fig. 4. First HPLC elution profile of active fraction.

Fig. 5. Second HPLC elution profile of active fraction.

20 Fig. 6. SDS-PAGE gel of both buccal saliva and submaxillary homogenate stained for glycoproteins.

Fig. 7. SDS-PAGE gel Coomassie stain of both buccal saliva and submaxillary homogenate.

Fig. 8. Capillary electrophoretogram of the isolated and purified shrew saliva peptide in sodium borate buffer.

Fig. 9. Capillary electrophoretogram of the isolated and purified shrew saliva peptide.

Fig. 10. Ultra-violet spectrum of the isolated and purified shrew saliva peptide.

Fig. 11. MALDI-TOF mass spectrum of the isolated and purified shrew saliva peptide.

Fig. 12. Peptide mass mapping of tryptic peptides of the isolated and purified shrew saliva peptide.

Fig. 13. MASCOT searching results of the MS/MS data from HPLC-ESI-Q-TOF analysis.

Fig. 14. Mealworms immediately post-injection and with total paralysis.

Detailed Description of the Invention

- 10 The invention involves isolation and purification of a peptide paralytic agent from shrew salivary gland or saliva (called "PS peptide" or soricidin). The peptide preferably has 54 amino acids and the sequence shown in Figure 1A (SEQ ID NO:1) or 1B (SEQ ID NO:2). The peptide may be isolated from any shrew having paralytic activity in its saliva, such as *Blarina*, *Neomys* and *Sorex* shrew species. The invention also includes a
- 15 bioassay using the common mealworm or other insect for rapid assessment of paralytic bioactivity. For example, the bioassay shows that paralytic saliva administered to the mealworm can keep it paralyzed but alive for at least 7 days. The toxin is very powerful; in dose response studies a 10 microlitre injection of 20% (w/v) crude gland extracts produces total paralysis in less than 1 sec while 10% requires 10 sec for total paralysis.
- 20 The 10 microlitre sample represented about 8 micrograms of total soluble extracted protein (0.8 mg/mL of extract, 0.010 mL of this injected = 0.008 mg = 8 micrograms total soluble protein). Of this, the peptide represents (as assessed from the gel stain density) about 1/10 of the protein in the whole extract (far right lane of gel picture). Thus, the actual peptide injected represents about 0.8 micrograms of material or 800 nanograms.
- 25 Using the bioassay and various chromatographic methods the inventors isolated a peptide(s) with a molecular weight of about 6000 (SDS- PAGE) that shows paralytic

activity. Unexpectedly, the small active component exists as part of a very high molecular weight, multiprotein complex (**Fig. 2; Fig. 3, lane 1**) the molecular weight of the complex was about 600,000 daltons. It appeared in a void volume fraction from a size exclusion column (Sephadex G-200) that has a molecular weight cut-off of 600,000 daltons. After purification, the complex shows a single band on the gel (**Fig. 3 lane 2**). The peptide sequence is readily obtained by known techniques, such as the standard sequential Edman degradation (P. Edman and G. Begg. 1967. Eur. J. Biochem. 1: 80-91. H.D. Niall, 1973. Methods Enzymol. 27: 942-1010.) and mass spectroscopic sequence determination.

Thus the invention includes a method of isolating and sequencing a paralytic shrew peptide by isolating the peptide as described in this application and sequencing the peptide. The sequence of the isolated and purified shrew saliva peptide is shown in **Figure 1A** (SEQ ID NO:1) or **Figure 1B** (SEQ ID NO:2) and the invention includes variants of the sequence as described herein. Two preferred methods are used to isolate the protein: i) size exclusion and ion exchange chromatography and ii) centrifugation through membranes with distinct molecular weight cut-offs: preferably 100,000, 10,000 and 3,000 molecular weight cut-off Centricons from Amicon other methods are also useful. The first method allows separation of the complexed active agent (very high molecular fractions) from where a free peptide of molecular weight 6000 would normally elute from the size exclusion chromatography column. The ion exchange chromatographic protocols employed a anion exchanger of a sodium phosphate buffer, neutral pH. The peptide is strongly bound to the complex (increased ionic strength does not dissociate it) and preferably is exposed to treatment with sodium dodecylsulfate (SDS) or with aqueous ethanol to dissociate it from the complex. Any short chain alcohol (preferably C1 to C6, more preferably C2 or C3) such as isopropyl alcohol, propanol or butanol may be used in place of ethanol. It appears that the bioactive peptide is kept complexed in the salivary gland until it is released as an active form in the saliva. The peptide isolate is weakly reactive with Clellands reagent indicating the presence of sulfhydryl groups and the amino acid cysteine although it is reasonable to expect these to exist in disulfhydryl bonds. The peptide preparation also showed an absorbance at 280

nm indicating the presence of aromatic amino acids. In particular, the peptide preparation showed weak absorption at 280 nm, but stronger absorption at 260 nm, indicating phenylalanine but not tyrosine and tryptophan. **Figure 14** shows mealworms immediately post-injection and with total paralysis.

- 5 The peptide may be modified as described below to produce variants of the paralytic peptide with different paralytic potencies. Some variants that will be developed by this process will have the potential to behave as competitive inhibitors (e.g. antidotes) to paralysis developed in response to our peptide.

Peptides of the Invention

- 10 The invention provides an isolated PS peptide. The term “PS peptide” as used herein includes the peptides shown in **Figure 1A** (SEQ ID NO:1) or **Figure 1B** (SEQ ID NO:2), homologs, analogs, mimetics, fragments or derivatives of the PS peptide.

- In one embodiment, the isolated PS peptide consists of 54 amino acid residues and has the sequence shown in **Figure 1A** (SEQ ID NO:1) or **Figure 1B** (SEQ ID NO:2). In
15 another embodiment, the PS peptide comprises sequences substantially identity to the above-noted peptides or comprising an obvious chemical equivalents thereof. It also includes peptide sequence plus or minus amino acids at the amino and/or carboxy terminus of the above-noted PS peptide sequences. In yet another embodiment, the invention includes fusion proteins, comprising the PS peptide, labeled PS peptides,
20 analogs, homologs and variants thereof.

- Within the context of the present invention, a peptide of the invention may include various structural forms of the primary PS peptide which retain biological activity. For example, a peptide of the invention may be in the form of acidic or basic salts or in neutral form. In addition, individual amino acid residues may be modified by oxidation
25 or reduction.

In addition to the full-length amino acid sequence, the peptide of the present invention may also include truncations, analogs and homologs of the peptide and truncations

thereof as described herein. Truncated peptides or fragments may comprise peptides of at least 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50 amino acids or more amino acid residues of the sequence listed above. Useful fragments also include, for example, 50-54, 45-50, 45-52, 44-55, 42-54, 40-54, 35-45 or 25-35 amino acids. Useful fragments are capable of providing analgesia or neuromuscular blocking. Amino acid nos. 42-54, 40-54, 38-54 and 45-54 are examples of useful fragments.

The invention further provides polypeptides comprising at least one functional domain or at least one antigenic determinant of a PS peptide.

Analogs of the protein of the invention and/or truncations thereof as described herein, may include, but are not limited to an amino acid sequence containing one or more amino acid substitutions, insertions, deletions and/or mutations. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids of the peptides of the invention with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent. Non-conserved substitutions involve replacing one or more amino acids of the amino acid sequence with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

One or more amino acid insertions may be introduced into the amino acid sequences of the invention. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging for example from 2 to 15 amino acids in length. For example, amino acid insertions may be used to destroy target sequences so that the peptide is no longer active. This procedure may be used to inhibit the activity of the peptide of the invention.

Deletions may consist of the removal of one or more amino acids, or discrete portions from the amino acid sequence of the PS peptide. The deleted amino acids may or may not be contiguous.

5 Analog of a protein of the invention may be prepared by introducing mutations in the nucleotide sequence encoding the peptide. Mutations in nucleotide sequences constructed for expression of analogs of a protein of the invention must preserve the reading frame of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures such as loops or hairpins, which could adversely affect translation of the mRNA.

10 Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

15 Alternatively, oligonucleotide-directed site-specific mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Deletion or truncation of a peptide of the invention may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by Sambrook et al (Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, 1989).

20 In addition, analogs of a protein of the invention can be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart). The peptides of the invention also include peptides having sequence identity to the PS peptide, mutated PS peptides and/or truncations thereof as described herein. Such peptides have amino acid sequences that correspond to nucleic acid sequences that hybridize under stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain a peptide of the invention. Peptides having sequence identity will often have the regions which are characteristic of the protein.

Peptides preferably have an amino acid sequence with at least 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, preferably 80-95% or more identity with the amino acid sequence of the PS peptide. The compound is optionally pharmaceutical grade purity (eg. for amino acids, this optionally means in excess of 99% purity, having a uniform crystalline structure, and white in color). Sequence identity is most preferably assessed by the BLAST version 2.1 program advanced search (parameters as above; Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403_410). BLAST is a series of programs that are available online at <http://www.ncbi.nlm.nih.gov/BLAST>. The advanced blast search (<http://www.ncbi.nlm.nih.gov/blast/blast.cgi?Jform=1>) is set to default parameters. (i.e. Matrix BLOSUM62; Gap existence cost 11; Per residue gap cost 1; Lambda ratio 0.85 default).

The invention also contemplates isoforms of the peptides of the invention. An isoform contains the same number and kinds of amino acids as a peptide of the invention, but the isoform has a different three-dimensional molecular structure. The isoforms contemplated by the present invention are those having the same properties as a peptide of the invention as described herein.

The present invention also includes a protein of the invention conjugated with a selected protein, or a selectable marker protein to produce fusion proteins. For example, the cDNA sequence to the PS peptide can be inserted into a vector that contains a nucleotide sequence encoding another peptide (e.g. GST-glutathione succinyl transferase). The fusion protein is expressed and recovered from prokaryotic (e.g. bacterial or baculovirus) or eukaryotic cells. The fusion protein can then be purified by affinity chromatography based upon the fusion vector sequence and the PS peptide obtained by enzymatic cleavage of the fusion protein.

An alternative method of producing the protein is by using a poly-histidine tag. The cDNA sequence is designed to have a poly-histidine tag on the N-terminal end. The protein is expressed in prokaryotic or eukaryotic cells, and then easily isolated using a nickel-affinity column. The polyhistidine (usually 6 histidines) adsorbs strongly to the

nickel attached to the affinity column while nothing else binds strongly. The 'his-tagged' peptide is isolated by washing the column with imidazole.

5 The proteins of the invention (including truncations, analogs, etc.) may be prepared using recombinant DNA methods. Accordingly, nucleic acid molecules of the present invention having a sequence that encodes a peptide of the invention are isolated using known technologies and are incorporated according to procedures known in the art into an appropriate expression vector that ensures good expression of the peptide. The cDNA is preferably obtained by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). The technology comes as a kit form. One isolates the messenger RNA that encodes the
10 peptide and then uses reverse transcriptase to convert all messengers in an extract of tissue to cDNA copies. One then amplifies the cloned DNA by standard PCR using a primer synthesized to match a segment of the peptide. The RACE technique is useful to obtain the full mRNA transcript since it codes for a series of peptides that are then cleaved after a bigger protein containing all of them is synthesized. Expression vectors
15 include but are not limited to cosmids, plasmids, or modified viruses (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses), so long as the vector is compatible with the host cell used. The expression "vectors suitable for transformation of a host cell", means that the expression vectors contain a nucleic acid molecule of the invention and regulatory sequences, selected on the basis of the host cells to be used for
20 expression, which are operatively linked to the nucleic acid molecule. "Operatively linked" means that the nucleic acid is linked to regulatory sequences in a manner that allows expression of the nucleic acid.

The invention therefore includes a recombinant expression vector of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the
25 necessary regulatory sequences for the transcription and translation of the inserted peptide-sequence. Suitable regulatory sequences are optionally derived from a variety of sources, including bacterial, fungal, or viral genes (For example, see the regulatory sequences described in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). Selection of appropriate regulatory

sequences is dependent on the host cell chosen, and may be readily accomplished by one of ordinary skill in the art. Examples of such regulatory sequences include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other sequences, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary regulatory sequences may be supplied by the native compound and/or its flanking regions.

The invention further provides a recombinant expression vector comprising a DNA nucleic acid molecule of the invention cloned into the expression vector in an antisense orientation. These vectors are useful experimental systems to study the peptides of the invention or its variants or to test antidotes. The peptides may or may not be toxic to the host cells. They are also useful to produce large amounts of the peptide. The vectors are particularly useful because insect-specific biological delivery agents (e.g. viruses) will provide immobilizing agents for specifically targeted insects. Viruses targeted against a specific insect pest are engineered to contain the gene fragment coding for the paralytic peptide along with expression regulatory sequences. The virus would target an insect species and then, during reproducing itself, also produce the peptide. Regulatory sequences operatively linked to the antisense nucleic acid can be chosen which direct the continuous expression of the antisense RNA molecule.

The recombinant expression vectors of the invention may also contain a selectable marker gene that facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of selectable marker genes are genes encoding a protein such as G418 and hygromycin which confer resistance to certain drugs, β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. Transcription of the selectable marker gene is monitored by changes in the concentration of the selectable marker protein such as β -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. If the selectable marker gene encodes a protein

conferring antibiotic resistance such as neomycin resistance transformant cells can be selected with G418. Cells that have incorporated the selectable marker gene will survive, while the other cells die. This makes it possible to visualize and assay for expression of recombinant expression vectors of the invention and in particular to determine the effect of a mutation on expression and phenotype. It will be appreciated that selectable markers can be introduced on a separate vector from the nucleic acid of interest.

The recombinant expression vectors may also contain genes which encode a fusion moiety which provides increased expression of the recombinant protein; increased solubility of the recombinant protein; and aid in the purification of a target recombinant protein by acting as a ligand in affinity purification. For example, a proteolytic cleavage site may be added to the target recombinant protein to allow separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein.

Recombinant expression vectors can be introduced into host cells to produce a transformed host cell. These cells are useful experimental systems. Accordingly, the invention includes a host cell comprising a recombinant expression vector of the invention. The term "transformed host cell" is intended to include prokaryotic and eukaryotic cells which have been transformed or transfected with a recombinant expression vector of the invention. Prokaryotic cells can be transformed with nucleic acid by, for example, electroporation or calcium-chloride mediated transformation. Nucleic acid can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofectin, electroporation or microinjection. Suitable methods for transforming and transfecting host cells can be found in Sambrook et al. (Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory press (1989)), and other such laboratory textbooks.

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells. For example, the peptides of the invention may be expressed in bacterial cells such as *E. coli*, *Pseudomonas*, *Bacillus subtilis*, insect cells (using baculovirus), yeast cells or

mammalian cells. Other suitable host cells can be found in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1991).

As an example, to produce PS peptides recombinantly, for example, *E. coli* can be used using the T7 RNA polymerase/promoter system using two plasmids or by labeling of plasmid-encoded proteins, or by expression by infection with M13 Phage mGPI-2. *E. coli* vectors can also be used with Phage lambda regulatory sequences, by fusion protein vectors (e.g. lacZ and trpE), by maltose-binding protein fusions, and by glutathione-S-transferase fusion proteins.

Alternatively, a PS peptide can be expressed in insect cells using baculoviral vectors, or in mammalian cells using vaccinia virus. For expression in mammalian cells, the cDNA sequence may be ligated to heterologous promoters and introduced into cells, such as COS cells to achieve transient or long-term expression. The stable integration of the chimeric gene construct may be maintained in mammalian cells by biochemical selection, such as neomycin and mycophenolic acid.

The PS DNA sequence can be altered using procedures such as restriction enzyme digestion, fill-in with DNA polymerase, deletion by exonuclease, extension by terminal deoxynucleotide transferase, ligation of synthetic or cloned DNA sequences, site-directed sequence alteration with the use of specific oligonucleotides together with PCR. For example, one to five or five to ten amino acids or more may be removed or mutated.

The cDNA sequence or portions thereof, or a mini gene consisting of a cDNA with an intron and its own promoter, is introduced into eukaryotic expression vectors by conventional techniques. These vectors permit the transcription of the cDNA in eukaryotic cells by providing regulatory sequences that initiate and enhance the transcription of the cDNA and ensure its proper splicing and polyadenylation. The endogenous PS gene promoter can also be used. Different promoters within vectors have different activities which alters the level of expression of the cDNA. In addition, certain promoters can also modulate function such as the glucocorticoid-responsive promoter from the mouse mammary tumor virus.

Some of the vectors listed contain selectable markers or neo bacterial genes that permit isolation of cells by chemical selection. Stable long-term vectors can be maintained in cells as episomal, freely replicating entities by using regulatory elements of viruses. Cell lines can also be produced which have integrated the vector into the genomic DNA. In this manner, the gene product is produced on a continuous basis.

Vectors are introduced into recipient cells by various methods including calcium phosphate, strontium phosphate, electroporation, lipofection, DEAE dextran, microinjection, or by protoplast fusion. Alternatively, the cDNA can be introduced by infection using viral vectors.

PS peptides may also be isolated from cells or tissues, including mammalian cells or tissues, in which the peptide is normally expressed.

The protein may be purified by conventional purification methods known to those in the art, such as chromatography methods, high performance liquid chromatography methods or precipitation.

For example, an anti-PS antibody (as described below) may be used to isolate a PS peptide, which is then purified by standard methods.

The peptides of the invention may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

Peptide Mimetics

The present invention also includes peptide mimetics of PS. "Peptide mimetics" are structures which serve as substitutes for peptides in interactions between molecules (See Morgan et al (1989), Ann. Reports Med. Chem. 24:243-252 for a review). Peptide mimetics include synthetic structures which optionally contain amino acids and/or peptide

bonds but retain the structural and functional features of a peptide, or enhancer or inhibitor of the invention. Peptide mimetics also include peptoids, oligopeptoids (Simon et al (1972) Proc. Natl. Acad, Sci USA 89:9367); and peptide libraries containing peptides of a designed length representing all possible sequences of amino acids corresponding to a peptide of the invention.

Peptide mimetics are designed based on information obtained by systematic replacement of L-amino acids by D-amino acids, replacement of side chains with groups having different electronic properties, and by systematic replacement of peptide bonds with amide bond replacements. Local conformational constraints can also be introduced to determine conformational requirements for activity of a candidate peptide mimetic. The mimetics may include isosteric amide bonds, or D-amino acids to stabilize or promote reverse turn conformations and to help stabilize the molecule. Cyclic amino acid analogues may be used to constrain amino acid residues to particular conformational states. The mimetics can also include mimics of inhibitor peptide secondary structures. These structures can model the 3-dimensional orientation of amino acid residues into the known secondary conformations of proteins. Peptoids may also be used which are oligomers of N-substituted amino acids and can be used as motifs for the generation of chemically diverse libraries of novel molecules.

Peptides of the invention are also useful to identify lead compounds for drug development. The structure of the peptides described herein can be readily determined by a number of methods such as NMR and X-ray crystallography. A comparison of the structures of peptides similar in sequence, but differing in the biological activities they elicit in target molecules can provide information about the structure-activity relationship of the target. Information obtained from the examination of structure-activity relationships can be used to design either modified peptides, or other small molecules or lead compounds that can be tested for predicted properties as related to the target molecule. The activity of the lead compounds is evaluated using assays similar to those described herein. Information about structure-activity relationships is obtained from co-crystallization studies. In these studies, a peptide with a desired activity is crystallized in

association with a target molecule, and the X-ray structure of the complex is determined. The structure is then optionally compared to the structure of the target molecule in its native state, and information from such a comparison is useful to design compounds expected to possess.

5 Therapeutic and Cosmetic Methods

The paralytic agent is useful for the neuromuscular disorder market including the well publicized cosmetic applications of neuromuscular blockers. (For a discussion of the use of Botox for immobilization of facial muscles and treatment of wrinkles, see: Fagien, S. 1999. *Plast Reconstr Surg* 103: 701-713; Carruthers, J, & Carruthers, A. 1998. *Dermatol Surg* 24: 1244-1247). Therapeutic applications of neuromuscular blockers such as relief of migraine, myofacial and other types of pain (i.e., analgesic activity) have recently been added to existing medical uses that include muscle tremors and neuromuscular diseases. New uses are steadily emerging including the cosmetic application of wrinkle reduction and, more recently, treatment of excessive sweating (also called hyperhidrosis; Blaheta, HJ, Vollert, B, Zuder, D, & Rassner, G. 2002. *Dermatol. Surg.* 28:666-671; Naumann, M & Hamm, H. 2002. *Br. J. Surg.* 89: 259-261).

Accordingly, in one embodiment, the present invention provides a method of blocking neuronal activity comprising administering an effective amount of PS peptide such as SEQ ID NO:1 or SEQ ID NO:2 or the other compounds described in this application to an animal in need thereof. The present invention also provides a use of an effective amount of a PS peptide as a neuromuscular blocker. The present invention further provides a use of an effective amount of a PS peptide in the manufacture of a medicament for blocking neuronal activity or providing analgesia.

Another embodiment of the invention provides a method of wound healing comprising administering an effective amount of PS peptide to an animal in need thereof. The present invention further provides a use of the PS peptide in wound healing., for example by providing analgesia For example, dressings can be embedded with the PS peptide or gels

containing the PS peptide can be applied to dressing, to behave as a long-lasting, local analgesic to wounds.

5 The phrase "substance that can block neuromuscular activity" as used herein includes all the peptides of the invention described herein that block neuromuscular activity temporarily or permanently, including but not limited to pain receptors (eg. a nociceptor, which is a peripheral nerve organ or mechanism for the reception and transmission of painful or injurious stimuli).

10 The term "effective amount" as used herein means an amount effective and at dosages and for periods of time necessary to achieve the desired result (e.g., blocking neuromuscular activity).

The term "animal" as used herein includes all members of the animal kingdom and is preferably mammalian, such as human. Administering a PS peptide or substance to an animal includes both *in vivo* and *ex vivo* administrations.

15 The term "a cell" as used herein includes a single cell as well as a plurality or population of cells. Administering a PS peptide or substance to a cell includes both *in vitro* and *in vivo* administrations.

20 The phrase "block neuromuscular activity" as used herein means that the substance can result in a decrease in neuromuscular activity as compared to a neuromuscular activity in the absence of the substance.

25 Blocking neuromuscular activity is useful for an analgesic in treating diseases such as migraine, tremors, neuromuscular disease, excess sweating and wrinkles. Accordingly, in a specific embodiment, the present invention relates to a method of treating the aforementioned diseases comprising administering an effective amount of a PS substance to an animal in need thereof. The present invention also provides a use of an effective amount of a substance that can block neuromuscular activity or provide analgesia. The present invention further provides a use of an effective amount of a PS substance that can

inhibit neuromuscular function to prepare a medicament to treat the aforementioned diseases.

As used herein, and as well understood in the art, "to treat" or "treatment" is an approach for obtaining beneficial or desired results, including clinical results. Beneficial or desired clinical results can include, but are not limited to, alleviation or amelioration of one or more symptoms or conditions, diminishment of extent of disease, stabilized (i.e. not worsening) state of disease or disorder, preventing spread of disease or disorder, delay or slowing of disease or disorder progression, amelioration or palliation of the disease or disorder state, and remission (whether partial or total), whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment.

Pharmaceutical and Cosmetic Compositions

The nucleic acids encoding the PS peptides are optionally formulated into a pharmaceutical composition for administration to subjects in a biologically compatible form suitable for administration *in vivo*. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the substance to be administered in which any toxic effects are outweighed by the therapeutic effects. The substances may be administered to living organisms including humans, and animals.

Administration of a therapeutically active amount of pharmaceutical compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of a substance may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation.

The polypeptide of the invention is preferably combined with other components such as a carrier in a composition such as a pharmaceutical composition or cosmetic composition. The compositions are useful when administered in methods of medical treatment, prevention, or diagnosis of a disease, disorder or abnormal physical state. For example, it may be administered as a neuromuscular blocker. They are useful for treatment of migraine, myofacial and other types of pain (analgesic function), muscle tremors and neuromuscular diseases, excessive sweating and wrinkles. They are also useful for wound healing by action as a local analgesic.

The pharmaceutical compositions can be administered to humans or animals by a variety of methods including, but not restricted to topical administration, oral administration, aerosol administration, intratracheal instillation, intraperitoneal injection, injection into the cerebrospinal fluid, intravenous injection and subcutaneous injection. Dosages to be administered depend on patient needs, on the desired effect and on the chosen route of administration. For example, the pharmaceutical compositions can be on a bandage, which is used for wound healing by acting as an analgesic. Nucleic acid molecules and polypeptides may be introduced into cells using *in vivo* delivery vehicles such as liposomes. They may also be introduced into these cells using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation or using liposomes.

The pharmaceutical compositions are prepared by known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the nucleic acid molecule or polypeptide is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA) or Handbook of Pharmaceutical Additives (compiled by Michael and Irene Ash, Gower Publishing Limited, Aldershot, England (1995)). On this basis, the compositions include, albeit not exclusively, solutions of the substances in association with one or more pharmaceutically acceptable vehicles or diluents,

and may be contained in buffered solutions with a suitable pH and/or be iso-osmotic with physiological fluids. In this regard, reference can be made to U.S. Patent No. 5,843,456.

On this basis, the pharmaceutical compositions optionally includes an active compound or substance, such as a peptide or nucleic acid molecule, in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and isoosmotic with the physiological fluids. The methods of combining the active molecules with the vehicles or combining them with diluents is well known to those skilled in the art. The composition optionally includes a targeting agent for the transport of the active compound to specified sites within tissue.

10 Preparation of antibodies

Antibodies to the peptide are useful to identify receptors and will find use in development of diagnostic tests. Some neuromuscular conditions result from malfunction of the peptide target. Detecting the target receptor molecules and determining their density on the surface of the cell, or their location on the cell surface is useful in diagnostics. This can be done with antibody treatment after peptide administration and then secondary detection of the antibody/peptide complexes as in the general ELISA protocol. Any method of labeling the peptide that would report on receptor density/location would be useful (e.g. radioactively labelled peptide or fluorescently tagged peptide). Once the peptide and its receptor are characterized as to how the effect is solicited, the PS peptide or variants are used to test how the target works in other tissues or animals or people. A variant or damaged receptor/target to the PS peptide or variant would not act in a manner that is identical to the characterized 'normal' target. The invention includes an isolated antibody immunoreactive with a polypeptide of the invention. Antibodies are preferably generated against epitopes of the sequence. The antibody may be labeled with a detectable marker or unlabeled. The antibody is preferably a monoclonal antibody or a polyclonal antibody. Such antibodies are employed to screen organisms. The antibodies are also valuable for immuno-purification of polypeptides from crude extracts. For example, one may contact a biological sample with the antibody under conditions allowing the formation of an immunological complex between the antibody and a

polypeptide recognized by the antibody and detecting the presence or absence of the immunological complex whereby the presence of the peptide of the invention or a similar peptide is detected in the sample. The invention also includes compositions preferably including the antibody, a medium suitable for the formation of an immunological complex between the antibody and a polypeptide recognized by the antibody and a reagent capable of detecting the immunological complex to ascertain the presence of the peptide of the invention or a similar polypeptide.

To recognize the peptide of the invention, one may generate antibodies against a range of unique epitopes throughout the molecule.

Monoclonal and polyclonal antibodies are prepared according to the description in this application and techniques known in the art. For examples of methods of the preparation and uses of monoclonal antibodies, see U.S. Patent Nos. 5,688,681, 5,688,657, 5,683,693, 5,667,781, 5,665,356, 5,591,628, 5,510,241, 5,503,987, 5,501,988, 5,500,345 and 5,496,705 that are incorporated by reference in their entirety. Examples of the preparation and uses of polyclonal antibodies are disclosed in U.S. Patent Nos. 5,512,282, 4,828,985, 5,225,331 and 5,124,147 which are incorporated by reference in their entirety.

The term "antibody" as used herein to includes fragments thereof which also specifically react with a PS peptide or fragments thereof. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments.

Chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region are also contemplated within the scope of the invention. Chimeric antibody molecules include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. Conventional methods are useful to make chimeric antibodies containing the immunoglobulin variable region which recognizes the PS peptide antigens of the

invention (See, for example, Morrison et al., Proc. Natl Acad. Sci. U.S.A. 81,6851 (1985); Takeda et al., Nature 314, 452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom patent GB 2177096B). It is expected that chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

Monoclonal or chimeric antibodies specifically reactive with a protein of the invention as described herein can be further humanized by producing human constant region chimeras, in which parts of the variable regions, particularly the conserved framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such immunoglobulin molecules are made by techniques known in the art, (e.g., Teng et al., Proc. Natl. Acad. Sci. U.S.A., 80, 7308-7312 (1983); Kozbor et al., Immunology Today, 4, 7279 (1983); Olsson et al., Meth. Enzymol., 92, 3-16 (1982)), and PCT Publication WO92/06193 or EP 0239400). Humanized antibodies can also be commercially produced (Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.)

Specific antibodies, or antibody fragments, such as, but not limited to, single-chain Fv monoclonal antibodies reactive against the peptides of the invention may also be generated by screening expression libraries encoding immunoglobulin genes, or portions thereof, expressed in bacteria with peptides produced from the nucleic acid molecules of PS peptides. For example, complete Fab fragments, VH regions and FV regions can be expressed in bacteria using phage expression libraries (See for example Ward et al., Nature 341, 544-546: (1989); Huse et al., Science 246, 1275-1281 (1989); and McCafferty et al. Nature 348, 552-554 (1990)). Alternatively, a SCID-hu mouse, for example the model developed by Genpharm, can be used to produce antibodies or fragments thereof.

The invention also includes methods of using the antibodies, such as in detection of receptors that bind to the peptide of the invention. For example, the invention includes a method for detecting the presence of a peptide of the invention by: a) contacting a

sample containing one or more peptides with an antibody of the invention under conditions suitable for the binding of the antibody to peptides with which it is specifically reactive; b) separating unbound peptides from the antibody; and c) detecting antibody which remains bound to one or more of the peptides in the sample.

5 **Research Tool**

The peptide and its derivatives are useful in research protocols to explore the neuromuscular junction and ion channels. The ability to selectively alter certain ion channels or classes of ion channels provides another tool with which to perturb the neuromuscular junction in a predictable manner. This identifies the role of susceptible peptide targets in neuromuscular functions and processes.

Examples

The following examples are illustrative embodiments and do not limit the scope of the invention.

Example 1: Isolation and purification of the shrew saliva peptide from the submaxillary saliva glands of the shrew (*Blarina brevicauda*)

Tissue Processing

The left and right submaxillary glands (ranging between 100 and 200 mg total weight) are dissected and placed into liquid nitrogen to flash freeze them. The tissue is crushed and powdered under liquid nitrogen. The tissue powder is quickly transferred to weighed receptacle and the weight of the transferred tissue powder is determined. The tissue is then homogenized in 50 mM potassium phosphate buffer, pH 7.0 to provide a 20% weight-to-volume (2 g/ 100 mL) homogenate. The homogenate is centrifuged at 12,000 x g at 4°C for 15 minutes to pellet the cell debris. The supernatant is removed.

If the glands are not to be used immediately they are flash frozen in liquid nitrogen and stored at -80°C or lower until processing.

Isolation of the peptide

5 The methods to isolate and purify the shrew saliva protein include: i) size exclusion and ion exchange chromatography (see **Figure 2 and 3**) and ii) centrifugation with distinct molecular weight cut-offs: preferably 100,000, 10,000 and 3,000 molecular weight cut-off Centricons from Amicon. The first method allows separation of the complexed active agent (very high molecular fractions) from where a free peptide of molecular weight
10 6000 would normally elute from the size exclusion chromatography column. The ion exchange chromatographic protocols employed a anion exchanger of a sodium phosphate buffer, neutral pH. The peptide is strongly bound to the complex (increased ionic strength does not dissociate it) and preferably is exposed to treatment with sodium dodecylsulfate (SDS) or with aqueous ethanol to dissociate it from the complex. Any short chain alcohol
15 (preferably C1 to C6, more preferably C2 or C3) such as isopropyl alcohol, propanol or butanol may be used in place of ethanol. Warming the crude extract at 40°C for 20 minutes increases the amount of isolatable peptide. It appears that the bioactive peptide is kept complexed in the salivary gland until it is released as an active form in the saliva. The peptide isolate is weakly reactive with Clellands reagent indicating the presence of
20 sulfhydryl groups and the amino acid cysteine although it is reasonable to expect these to exist in disulfhydryl bonds. The peptide preparation also showed a weak absorbance at 280 nm and a strong absorbance at 260 nm indicating the presence of phenylalanine, but not tryptophan and tyrosine.

The size exclusion method can also include a precipitation step of the soluble proteins
25 with cold acetone (-20°C or -80°C; 10:1 v/v acetone: homogenate), which also precipitates the larger molecular weight proteins. This acetone precipitation step can be done before or after size-fractionation. The acetone precipitate is air dried rapidly and is active for long period of time (Ellis S & Krayner O (1955) J Pharm Exper Therapeutics 114: 127-137). The precipitate (~ 50 mg) is dissolved in about 1 mL of 25 mM potassium

phosphate buffer and isolated by HPLC immediately or first incubated at 40°C before HPLC isolation.

High Pressure Liquid Chromatographic Isolation.

5 A way to isolate the peptide once the acetone precipitate is re-dissolved is reversed phase HPLC. A Phenomenex Jupiter C-18 column, 250 x 4.6 mm, 5 u at 20 -25 °C and a gradient elution from 10 % (v/v) acetonitrile: 90 % (v.v) water to 60 % acetonitrile: 40% water are used over 30 minutes and a flow rate of 1.0 mL/min. All solvents contain 0.1% (v/v) trifluoroacetic acid (TFA). This provide the elution profile shown in Figure 4.

10 The active fraction of this first HPLC set is that peak eluting at about 14.7 minutes. This peak is collected (See Fig 4, bar on 'time axis'). This material is lyophylized overnight to remove solvents and TFA.

The residue containing the main peptide of interest and 2 to 3 minor other proteins is dissolved in a minimum volume of 25 mM potassium phosphate buffer. The solubilized peptide is purified under another HPLC protocol. A Phenomenex Jupiter C-18 column, 15 250 x 4.6 mm, 5 u at 20 -25 °C and a gradient elution from 10 % (v/v) acetonitrile: 90 % (v.v) water to 60 % acetonitrile: 40% water is used over 40 minutes and a flow rate of 2.3 mL/min. All solvents contain 0.1% (v/v) trifluoroacetic acid (TFA). This provides the elution profile shown in (See Fig HPLC 02) with peptide eluting at 13.76 min: the collected eluant shown by the solid bar on the 'time axis' is pure peptide. This material is 20 lyophylized removing the solvents and the TFA. This material is pure peptide by HPLC (Figure 5), by SDS-PAGE (Figures 6 and 7) and by CE (Figures 8 and 9).

Capillary Electrophoresis

Purified shrew saliva peptide (dissolved in 25 mM potassium phosphate buffer, pH 7.0) was subjected to capillary electrophoresis using Beckman Coulter P/ACE Capillary 25 Electrophoresis System in a 60 cm fused silica column ((75 um internal diameter, 375 um outer diameter) with sodium borate buffer (1 Molar, run buffer) thermostated to 25°C. The voltage regime was a 0.17 minute ramp to 30,000 volts for 20 minutes at normal

polarity. The injection pressure was 0.5 pounds per square inch for 10.0 seconds providing a sample volume of approximately 5 nL (nanolitres)).

Figure 8 shows the electrophoretogram of the purified peptide in buffer and had an elution time of 2.67 minutes. **Figure 9** shows an identical electrophoretic run of the 25 mM potassium phosphate buffer. This peak showed an identical uv-spectrum as that obtained with a standard spectrophotometer (see below).

Electronic Spectrum

Purified peptide was dissolved in 50 mM potassium phosphate, pH 7.0 and its ultra-violet spectrum measured (**Figure 10**). The spectrum showed no absorbtion in the 280 nm range and thus indicated that the amino acids tryptophan and tyrosine were not present in the peptide. The shoulder centred about 260 nm indicated the presence of the amino acid phenylalanine while the low absorbtion indicated only a small amount of the amino acid present in the peptide. Subsequent amino acid sequencing of the peptide was consistent with this as there was no tryptophan or tyrosine and only one phenylalanine residue detected.

Post-translational modification

Many salivary proteins are modified post-translationally by glycation but the isolated and purified shrew saliva peptide is not a glycoprotein produced by such a process. Shrew saliva peptide does not have carbohydrates attached covalently to its structure.

SDS-PAGE

Figure 6 shows an SDS-PAGE (15 % acrylamide) gel of both buccal saliva (mouth wash) and sub-maxillary homogenate along with internal standards of a non-glycosylated and a glycosylated protein. **Figure 7** shows a protein stain (Coomassie) after the glycostaining was complete and is the same gel restained with Coomassie. Shrew saliva peptide appears at as the most mobile of the proteinaceous components (lowest stained,

diffuse band) and did not react positively to the glycostain as did other proteins in these biological fluids at larger molecular weight.

Example 2: Amino Acid Sequence

5 The purified peptide was subjected to N-terminal sequencing using the Edman sequential degradation (**Figure 1B**). Mass spectroscopy/mass spectroscopy (ms/ms) was used to obtain C-terminal amino acids (**Figure 1A**). Both sequences are useful peptides.

Molecular Ion of the purified peptide

10 The molecular mass of isolated and purified shrew saliva peptide (Bruker Reflex III, MALDI-TOF; Linear mode, HCCA matrix, two layer method) provided a molecular cation (MH⁺) of 5805.8 Daltons and thus a molecular mass (M) of 5804.8 Daltons. (See **Figure 11**)

Tryptic digest peptide mass map

15 The tryptic digest followed by peptide mass mapping by MALDI-TOF provided the mass spectrogram presented in **Figure 12**. This digestion mass map is absolutely distinctive of isolated and purified shrew saliva peptide. There were no matches of this mass map in and public database using the MASCOT searching (**Figure 13**) (Perkins et al. 1999. Electrophoresis, 20(18) 3551-3567).

Theoretical isoelectric points and mass

20 The theoretical isoelectric point and mass of the isolated and purified shrew saliva protein can be calculated. The theoretical isoelectric point is 4.60. Its theoretical mass is 5754.51 Da with a reduction of 6 mass units because of disulfide bond formation it would be 5748.5 Da. The mass shortfall from the mass spectrometry determined molecular ion is (5804.8-5748.5) 56.3 Da, and may represent 3 integral water molecules (3 x 18 = 54). If the histidine residue is protonated this would provide another mass unit giving a mass
25 discrepancy of 1 mass unit.

Example 3: Bioassay

The invention includes a bioassay that shows that paralytic saliva administered to the mealworm can keep it alive for at least 7 days. **Figure 14** shows mealworms immediately postinjection and with total paralysis. Other insects are also useful in the bioassay.

5 Example 4: Toxicity

Table 1 shows the toxicity of general crude extract (10 microliters per 100 mg worm mass). **Table 2** shows the toxicity of the preparation during purification procedure (5 microliters injection per 100 milligram of worm).

10 While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

15 All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Toxicity studies

TABLE 1

A. General Crude extract toxicity (10 microliters per 100 mg worm mass)

Homogenate concentration*	# injected worms	# paralyzed worms	Average time to paralysis	Comment
20%	5	5	< 1 second	Instant paralysis
10%	6	6	10 seconds	
5%	9	6	7 sec	not paralyzed extremely laboured movement
1%	5	2	53 sec	laboured movement
20% (boiled for 5 min)	5	0	not applicable	no symptoms

* equivalent to 2 gm tissue homogenized in 100 mL buffer

TABLE 2

B. Toxicity of preparation during purification procedure (5 microliters injection per 100 milligram of worm)

Sample Injected	# injected worms	# paralyzed worms	Average time to first noted effect	Amount protein in injection
20%	5	5	<1 sec	65 ug
Acetone ppt of high molec. wt.	5	4	<3 sec	55 ug
Warmed supernat.	5	5	~5 sec	45 ug
Purified peptide	6	5	10 sec	0.8 ug
Saline	5	0	not applicable	0 ug

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